

Heterogeneity of Wild *Leishmania major* Isolates in Experimental Murine Pathogenicity and Specific Immune Response

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Virulence variability was investigated by analyzing the experimental pathogenicity of 19 *Leishmania major* strains in susceptible BALB/c mice. Twelve strains were isolated from Tunisian patients with zoonotic cutaneous leishmaniasis; seven strains were isolated in Syria ($n = 1$), Saudi Arabia ($n = 2$), Jordan ($n = 2$), or Israel ($n = 2$). BALB/c mice were injected in the hind footpad with 2×10^6 amastigotes of the various isolates, and lesion progression was recorded weekly for 9 weeks. Interleukin-4 (IL-4) and gamma interferon (IFN- γ) production of lymph node mononuclear cells activated in vitro with parasite antigens were evaluated 5 weeks after infection. We show that disease progression induced by different *L. major* isolates was largely heterogeneous although reproducible results were obtained when using the same isolate. Interestingly, isolates from the Middle East induced a more severe disease than did the majority of Tunisian isolates. Strains with the highest virulence tend to generate more IL-4 and less IFN- γ in vitro at week 5 postinfection as well as higher levels of early IL-4 mRNA in the lymph node draining the inoculation site at 16 h postinfection. These results suggest that *L. major* isolates from the field may differ in virulence, which influences the course of the disease induced in mice and the type of immune response elicited by the infected host.

Zoonotic cutaneous leishmaniasis (ZCL) caused by *Leishmania major* is endemic in North Africa and the Middle East. In these regions, ZCL is a major public health problem, especially in rural areas where it may cause considerable morbidity (54). ZCL is a polymorphic disease which may express various clinical patterns, ranging from asymptomatic infection featuring a conversion of the leishmanin skin test without apparent lesion to benign self-limited cutaneous sore(s) or to more protracted and extensive lesion(s), which may cause severe disfiguring. This clinical polymorphism, which may be observed even within small endemic foci, may reflect either variability in the host immune response and/or variability in the parasite virulence. These two parameters can also be modified by factors linked to the vector, as the sandfly saliva, for instance (43, 50). Because these factors are most likely intricately in human infection, they have been difficult to unravel. By contrast, the experimental model of leishmaniasis that makes use of genetically defined inbred mice, infected under controlled conditions, has proved a powerful tool to study some of the host parameters associated with susceptibility or resistance. The importance of genetic factors in shaping the innate or acquired immunity of the host to *L. major* parasites has been demonstrated (9, 15, 22). Thus, factors that determine the development of a TH1 response are associated with resistance, whereas factors that orient the immune response toward a TH2 dominance are associated, as in BALB/c mice, with protracted infection and visceralization (14, 27, 45).

Taking advantage of the homogeneity of the genetic background and of the immune response developed by an inbred

mouse strain (i.e., BALB/c mice), the murine model of leishmaniasis is also suitable for comparing the intrinsic virulence of different isolates of *Leishmania*. In this case, variations in virulence between parasite strains feature variations in the course of the experimental infection, as has already been reported by Almeida and colleagues in comparing the pathogenicity of wild *Leishmania amazonensis* strains isolated from patients with cutaneous versus mucosal or visceral leishmaniasis (1).

Early studies have shown that *L. major* promastigotes, grown in vitro, are pathogenic to BALB/c mice only when collected at the stationary phase as metacyclic parasites (7, 42). It is also well known that promastigotes maintained in culture for long periods frequently lose their capacity to induce disease in BALB/c mice but may shift back to virulence when passaged in vivo or when used as amastigotes (12, 55). Similar observations were also reported when using avirulent clones derived from virulent strains (25, 48). All these experiments have allowed the identification of components of the parasite playing key roles in its biology and hence in its infectivity. Changes in surface glycoconjugates, mainly lipophosphoglycan (LPG), were shown to correlate with changes in infectivity (38, 40, 41). Other virulence factors have been identified by mutagenesis or knockout experiments (5, 10, 34, 47, 56).

The studies reported above have mainly focused on laboratory-manipulated parasite strains but not on recently established wild isolates. It is therefore unknown whether the conclusions on virulence attenuation are relevant to the biology and natural pathogenicity of the parasite in the field.

In the present study, pathogenicity of different *L. major* isolates was evaluated in the mouse experimental model of leishmaniasis. BALB/c mice were infected with amastigotes generated from 19 strains of *L. major* isolated in Tunisia (North Africa) or in some countries of the Middle East. The

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TABLE 1. Characteristics of the *L. major* isolates used in this study

Isolate	Zymodeme	Country of origin
MHOM/TN/94/GLC03	MON-25	Tunisia
MHOM/TN/94/GLC07	MON-25	Tunisia
MHOM/TN/94/GLC09	MON-25	Tunisia
MHOM/TN/94/GLC22	MON-25	Tunisia
MHOM/TN/94/GLC25	MON-25	Tunisia
MHOM/TN/94/GLC27	MON-25	Tunisia
MHOM/TN/95/GLC32	MON-25	Tunisia
MHOM/TN/95/GLC34	MON-25	Tunisia
MHOM/TN/95/GLC36	MON-25	Tunisia
MHOM/TN/95/GLC67	MON-25	Tunisia
MHOM/TN/95/GLC94	MON-25	Tunisia
MHOM/TN/88/TN435	MON-25	Tunisia
MHOM/SY/94/Abdou	MON-26	Syria
MHOM/JO/95/JCL-83	MON-103	Jordan
MHOM/JO/95/JCL-85	MON-103	Jordan
IPAP/IL/84/LRC-L465	MON-68	Israel
MHOM/IL/67/Jericho-II	LON-70	Israel
MHOM/SA/-/No77	MON-26	Saudi Arabia
MMER/SA/-/No99	MON-26	Saudi Arabia

patterns of disease they cause were analyzed by using clinical, parasitological, and immunological criteria. We demonstrate strain differences in disease progression correlating with differences in in vitro parasite growth and some parameters of the host immune response, which may reflect virulence variations between these wild strains.

MATERIALS AND METHODS

Animals. BALB/c mice were purchased from the IFFA CREDO Laboratory (Lyon, France) and locally bred.

Parasites. Nineteen *L. major* isolates were used in this study (Table 1). Twelve strains originated in Tunisia and seven in the Middle East. Eleven Tunisian *L. major* isolates were collected during a prospective study of ZCL conducted in 1994 at El Guettar, southern Tunisia (28), and one strain was isolated in 1988 from a human case of ZCL at Kairouan, central Tunisia. All these isolates were identified as *L. major* by recombinant DNA probes (13) and typed as *L. major* zymodeme MON-25 by isoenzyme analysis (kindly performed by Jean-Pierre Dedet and Francine Pratlong from the Centre Nationale de Référence sur les leishmanioses, Service d'identification enzymatique des *Leishmania*, Montpellier, France). Seven Middle Eastern *L. major* isolates were also used in this study. They were from Syria ($n = 1$; isolated from a human and kindly provided by Riadh Ben-Ismaïl, Laboratoire d'Epidémiologie et d'Ecologie parasitaire, Institut Pasteur de Tunis), Jordan ($n = 2$; isolated from human zoonotic cutaneous lesions and kindly supplied by Chaden Kamhaoui, Yarmouk University, Irbid, Jordan), Israel ($n = 2$; one isolated from the vector *Phlebotomus papatasi* in Wedi Uvda, South West Israel [sixth passage from Navy-Nicoll-MacNeal medium], and one isolated from a patient infected in Jericho in the Jordan Valley [second passage from an experimental infection of the reservoir *Psammomys obesus*]; both strains were kindly provided by R. L. Jacobson, Department of Parasitology, Hebrew University-Hadassah Medical School, Jerusalem, Israel), and Saudi Arabia ($n = 2$; one isolated from the reservoir *Meriones libicus* and the other from a 26-year-old Filipino patient having three lesions with secondary infections. These strains were generously provided by A. Ibrahim Elfeki and S. A. El Amri, Leishmaniasis Administration, Ministry of health, Saudi Arabia Kingdom).

Parasite culture and antigens. For mass culture, promastigotes were grown on NNN medium at 26°C and then progressively adapted to RPMI 1640 medium (Sigma, St. Louis, Mo.) containing 2 mM L-glutamine, 100 U of penicillin/ml, 100 µg of streptomycin/ml, and 10% heat-inactivated fetal calf serum (FCS) (complete medium); parasites were then collected at the stationary growth phase. For kinetics of growth in culture, promastigotes were taken at log-phase culture in complete medium, adjusted to 10^6 parasites/ml in a constant volume, and incubated at 26°C. The growth rate was monitored by daily enumeration of promastigotes/ml over 6 days. For in vitro cytokine induction, leishmanial total antigens (LTA) were prepared according to Melby et al. (31) from a frozen-and-thawed preparation of *L. major* promastigotes. Amastigotes were obtained after one

passage into BALB/c mice and harvested from skin lesions after differential centrifugation.

Experimental infection in mice. Amastigotes or stationary phase promastigotes were inoculated subcutaneously into the left hind footpad of 5- to 6-week-old BALB/c mice at a dose of 2×10^6 parasites in 50 µl of saline (53). Each parasite preparation was injected into a group of five mice. Observations of the inoculation sites were made at weekly intervals, and footpad swelling was measured in millimeters by a Vernier caliper. The lesion size was defined as the increase in the footpad thickness after subtracting the size of the controlateral uninfected footpad.

In vitro generation of cytokines and cytokine assays. Five weeks after inoculation of *L. major* parasites, mice were killed and popliteal lymph nodes draining the cutaneous lesions were removed and disrupted into cellular suspension at 4×10^6 cells/ml. Cells were cultured in 24-well Costar plates (Nunc, Roskilde, Denmark) in complete medium, alone or in the presence of a frozen-and-thawed preparation of *L. major* LTA (equivalent of 2×10^6 promastigotes/ml). Supernatants were harvested after 48 h (for interleukin-4 [IL-4] measurement) or 72 h (for gamma interferon [IFN-γ] measurement) of culture at 37°C with 5% CO₂ and stored at -80°C for later assay. Quantitation of IL-4 and IFN-γ levels was performed by enzyme-linked immunosorbent assay (ELISA). ELISA flat-bottomed plates (Nunc) were coated overnight at 4°C with 50 µl of purified rat monoclonal antibodies to murine IL-4 (2 µg/ml) and IFN-γ (4 µg/ml) (PharMingen, San Diego, Calif.) diluted in 0.1 M carbonate-bicarbonate buffer (pH 8.2). Excess coating buffer was flicked off and nonspecific binding sites were blocked with 0.5% gelatin in phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBS-T-G) for 2 h at room temperature. After five washes with 0.1% Tween 20 in PBS, 100 µl of the culture supernatants diluted 1:4 in PBS-T-G were dispensed into the appropriate wells and incubated overnight at 4°C. For calibration of the assays, recombinant cytokines (PharMingen) were incubated in serial dilutions. Unbound supernatant was washed off five times as above, and biotinylated rat monoclonal antibodies (PharMingen) to murine IL-4 (0.1 µg/ml) and IFN-γ (0.2 µg/ml) were added, followed by incubation for 45 min at room temperature. Unbound conjugate was washed off six times before adding 1 µg of streptavidin-peroxidase (AMDEX; Amersham Pharmacia Biotech, Buckinghamshire, England). Incubation was done at room temperature for 30 min. One hundred microliters of orthophenylene diamine (Sigma) was added at 1 mg/ml in 0.1 M citrate buffer (pH 5) containing 0.03% hydrogen peroxide. The plates were incubated 10 min at room temperature in the dark. Reaction was stopped with 50 µl of sulphuric acid 4N (H₂SO₄). Optical density was measured with an ELISA reader (Titertek Multiskan, Helsinki, Finland). Using the standard curves, the respective cytokine concentrations were determined. The detection limits in these assays were 40 and 80 pg/ml for IL-4 and IFN-γ, respectively.

In vitro Leishmania infection of murine macrophages. Murine bone marrow macrophages (MBMM) were obtained from bone marrow extruded from the femur and tibia of female BALB/c mice. MBMM were cultured in multichambered slides at 1.5×10^5 cells/well in 500 µl of complete medium. To stimulate MBMM growth and maturation, culture medium was further supplemented with 20% L-929 fibroblast-conditioned medium, as a source of macrophage colony stimulating factor. After 6 days of culture at 37°C in 5% CO₂, medium was removed, MBMM were washed and new RPMI with 10% FCS but lacking L-929 fibroblast-conditioned medium was added. Intraleisional amastigotes were purified from nonulcerated lesions by differential centrifugation and counted using the vital stain trypan blue. These parasites were used to infect MBMM at a final ratio of four parasites per one macrophage. Two hours after the addition of parasites, the macrophages were rinsed five times with PBS to eliminate nonphagocytized amastigotes. Cultures were then incubated at 37°C in 95% air and 5% CO₂. Experiments were done at different time points: 30 min and 2, 24, and 72 h. At the time indicated, wells were rinsed with PBS, the coverslips were removed, and infected macrophages were fixed with ethanol for 1 h at room temperature. Slides were then washed and stained with Giemsa to monitor the infection.

Enumeration of infected macrophages was performed at the center of the slide chamber, where cells are fully spread and parasites could be easily enumerated. At this level of the slide the parasite/macrophage ratio may be higher than four.

Total RNA extraction. Infected mice (BALB/c and C57BL/6) were sacrificed 16 h after infection, and popliteal lymph nodes draining the lesions were collected. Lymph nodes were homogenized in Trizol reagent (Gibco-BRL, Life Technologies, Paisley, Scotland), and total RNA was isolated according to the manufacturer's recommendations. Briefly, after cell lysis, RNA was precipitated with isopropanol, washed with 70% ethanol, and solubilized in sterile water. RNA was treated with RNase-free DNase (Boehringer, Mannheim, Germany) to eliminate any genomic DNA contamination.

RT and PCR. First-strand cDNA synthesis was performed on approximately 5 µg of RNA in a total volume of 20 µl containing 1 µg of random-hexamer

primers (Pharmacia Biotech, Inc.), 1× first-strand buffer (50 mM Tris-HCl [pH 8.3], 75 mM KCl, 5 mM MgCl₂; Gibco-BRL, Gaithersburg, Md.), 15 mM dithiothreitol (Gibco-BRL), and 500 μM deoxynucleoside triphosphate mixture (Pharmacia Biotech). The mixture was heated at 65°C for 5 min. The tube was quenched on ice, and the content was pulsed in a microfuge at 4°C. Two hundred fifty units of Moloney murine leukemia virus reverse transcriptase (RT) (Amersham Life Science, Cleveland, Ohio) and 1.5 U of RNasin RNase inhibitor (Promega, Madison, Wis.) were added, and the 20-μl reaction was incubated at 42°C for 1 h to remove any residual RNA from the reaction product. The reaction was stopped by heating to 95°C for 5 min. One microliter of diluted first-strand cDNA was amplified 30 cycles (Perkin-Elmer, Foster City, Calif.) in a reaction containing 1× PCR buffer (1 mM Tris-HCl [pH 9.0], 5 mM KCl, 0.01%, 1.5 mM MgCl₂) (Amersham Life Science), 0.6 μM concentrations of each sense and antisense primer (Genset, SA, Paris, France), 200 μM deoxynucleoside triphosphate mixture (Pharmacia Biotech), 0.5 U of *Taq* DNA polymerase (Amersham Life Science), and sterile water to 25 μl. Cycling conditions were as follows: 95°C for 3 min, 50°C for 2 min, and 70°C for 2 min for 1 cycle, followed by 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min for a total of 30 cycles. A final extension of 72°C for 6 min was included. PCR products were visualized by UV light after electrophoresis through a 1 to 1.5% agarose gel containing 0.5 μg of ethidium bromide/ml. Nucleotide sequences of primers for IL-4 and the constitutively expressed gene hypoxanthine phosphoribosyltransferase (HPRT) were synthesized by Genset (Genset, SA): HPRT forward (5'-GACAGGACTGAAA GACTTGC-3'), HPRT reverse (5'-GTTGAGAGATCATCTCCACC-3'), IL-4 forward (5'-CAGAGCTATTGATGGGTCTC-3'), and IL-4 reverse (5'-TTCCA GGAAGTCTTTCAGTG-3'). The HPRT gene is commonly used in semiquantitative RT-PCR to assess the relative efficiency of each individual PCR. The level of IL-4 mRNA was normalized between samples with the level of HPRT message used as a positive-control standard, and relative levels were compared between different groups of infected mice. To confirm that amplification was based solely on cDNA, PCR using primers for HPRT was performed on non-reverse-transcribed total RNA for each sample.

Parasite genes expression. *Leishmania*-specific mRNA gene expression was assessed by RT-PCR analysis. Comparisons were made between five *L. major* strains, expressing various levels of pathogenicity, at both stage amastigotes and stationary-phase promastigotes. Briefly, total RNA of the parasites was prepared as previously described. mRNA transcripts were purified using a poly(A)⁺ RNA isolation kit (us72700 Amersham). PCRs were conducted in the presence of the corresponding specific gene primers at the cycling conditions described above. The gene-specific primer pair sequences used in PCR are shown in Table 2. The level of mRNA was normalized between samples, with the level of α-tubulin message used as a positive-control standard.

LACK sequencing. The total RNA from four *L. major* isolates was reverse transcribed and amplified by PCR. Specific oligonucleotide primers of LACK peptide encompassing amino acids 155 to 196 (forward, 5'-TGTGGGACCTGC GCAATGGC-3'; reverse, 5'-CTGTGGCCCTTGAGCGTGCG-3') synthesized by GENSET were used to determine the double-stranded cDNA sequence of LACK. Dideoxynucleotide sequencing reactions were performed using the ABI PRISM dye terminator-cycle sequencing chemistry and resolved on an ABI 377 DNA sequencer (ABI Perkin-Elmer). Individual cDNA sequences from both strands were assembled into complete contiguous sequences. Sequence analysis was performed by multiple-sequence alignment using McDNASIS software.

Statistical analysis. Reliability of the measurement of the footpad lesions was assessed by a test-retest technique and by the comparison of the mean response and its 95% confidence limits for each measurement. Correlation between continuous variables (parasite load; lesion size) was tested by bivariate scatter plots. One-way analysis of variance was performed in order to evaluate the heterogeneity of different *L. major* isolates according to the size of lesions induced in the BALB/c mice. Fisher's exact χ^2 test permitted comparison of the proportion of macrophages infected by each isolate. Nonparametric statistics were used in order to compare cytokine expressions that were measured on a subsample of isolates. All these statistical techniques were performed by BMDP statistical software BMDP Statistical Software, Inc., Los Angeles, Calif.).

RESULTS

Pathogenicity of *L. major* isolates in BALB/c mice. Preliminary studies using promastigotes collected at the stationary phase and derived from 12 wild strains of *L. major* (11 from Tunisia and 1 from Syria) showed significant and reproducible differences in the pathogenicity induced in BALB/c mice which

TABLE 2. Gene-specific and other primer pair sequences used in PCR

Gene or other	Primer pair sequences ^a
α-Tubulin.....	5'-ATGCGTGAGGCTATCTGCATC-3' 5'-GTCAGCACGAAGTGGATGCGC-3'
<i>lpg-1</i>	5'-GCCCTCGAGGCTGGGGTCAACAGAAAGTC-3' 5'-GCCGAATTCGATGGCGCCGCTCGCTGG-3'
<i>lpg-2</i>	5'-GCCCTCGAGCTCAGATTGGAAGTGC-3' 5'-GCCCATATGAACCACTACTCGCTCTGTG-3'
Gene B.....	5'-GCCCTCGAGGTTGCCGCGAGCGTGCTC-3' 5'-GCCCATATGGGAAGCTCTTGCACG-3'
Clp B.....	5'-GCCGCGGCGCGAGGCTGTGTCGGTCTC-3' 5'-GCCCATATGACGACGACGAGCCAG-3'
<i>Cpb</i>	5'-GCCGCGGCGCGCACGTAAGTGGCAAATGGC-3' 5'-GCCCATATGGCGACGTCGAGGGCC-3'
<i>Cpc</i>	5'-GCCCTCGAGCTGCGCGGGTATGCCAGC-3' 5'-GCCGAATTCGATGGCCCTCCGCGCCAAG-3'
KMP-11.....	5'-GCAGCTCGGCGAACTTCTGC-3' 5'-TCGGCGAAGCTGGAGCGCCT-3'
LACK.....	5'-TCACCTCCCTGGCCTGCCCG-3' 5'-CGGCCCGCGGACACGATCAG-3'

^a For each primer pair, the forward primer is listed first and the reverse primer is listed second.

correlate with parasite load (data not shown). Considering that promastigotes, grown in vitro, are heterogeneous populations even when collected at the stationary phase and in order to rule out the possibility that variation in the pathogenicity displayed by promastigotes could be generated by variation in the efficiency of in vitro metacyclogenesis, experimental infections were performed using the amastigote form of the parasite instead of promastigotes. The experimental protocol also included additional *L. major* isolates from the Middle East.

The pathogenicity of 19 *L. major* isolates (12 from Tunisia and 7 from the Middle East) was tested in BALB/c mice. Mice were inoculated subcutaneously in the hind footpad with 2 × 10⁶ amastigotes. Figure 1 shows the kinetics of lesion development induced in groups of mice (five mice per group) infected with amastigotes derived from the Tunisian strains (Fig. 1A) and the Middle Eastern strains (Fig. 1B).

A large heterogeneity in the evolution of the experimental disease induced by the different strains was observed. Some strains (MHOM/TN/95/GLC32 and MHOM/TN/88/TN435) induced only small lesions that progressed slowly without exceeding 1 mm by week 5 with a slight increase thereafter. Other strains showed a gradual but steady increase of lesion size, generally stabilizing between 2 and 3 mm around weeks 7 to 9 postinoculation.

The experimental infections were repeated twice using two independent preparations of the *L. major* isolates containing the same parasite dose of the amastigote stage and revealed a high reproducibility of the results. Interestingly, the majority of the Tunisian strains induced in mice a less severe disease than did the majority of the Middle Eastern isolates. The differences

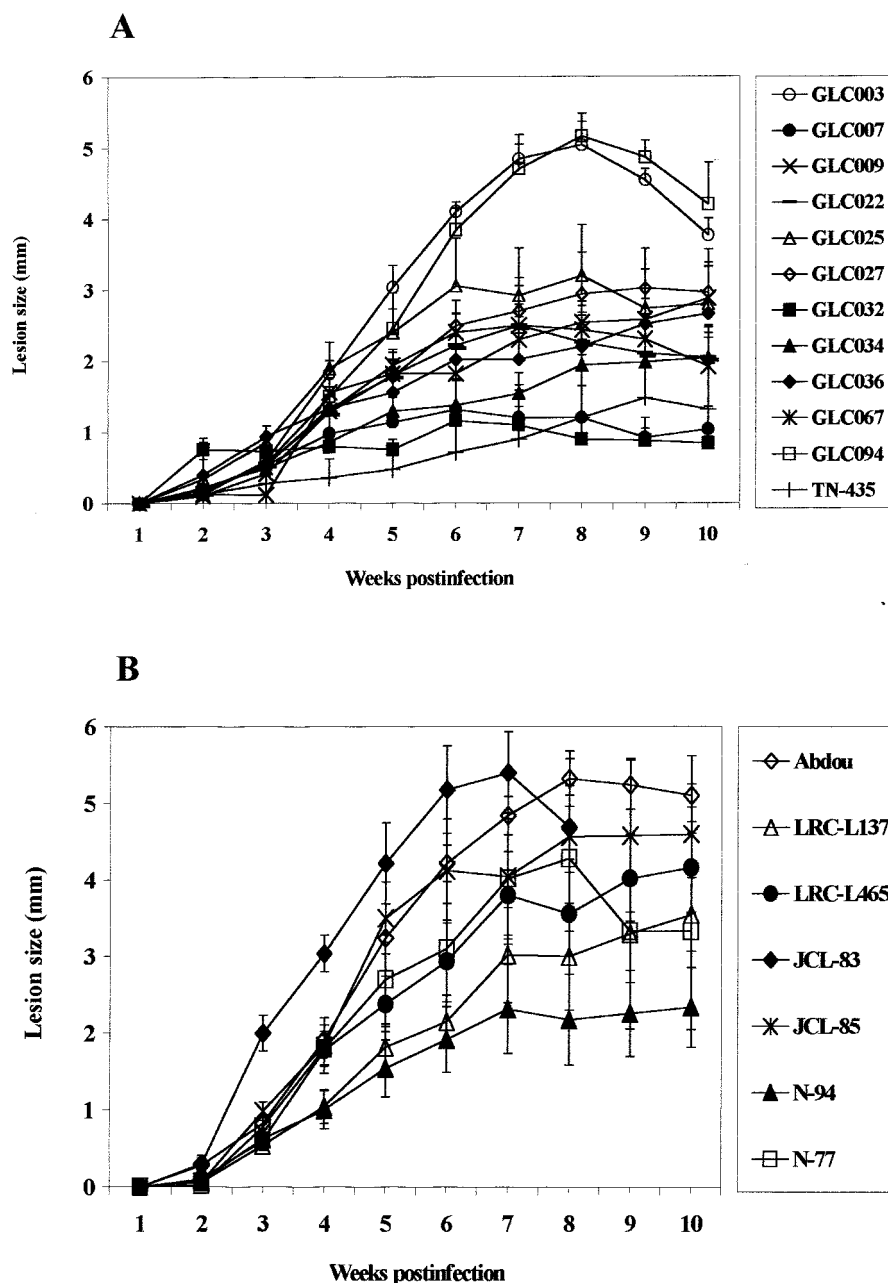


FIG. 1. Course of lesion development in BALB/c mice inoculated with amastigotes derived from 12 *L. major* isolates from Tunisia (A) or 7 isolates from the Middle East (B). Mice were inoculated subcutaneously with 2×10^6 amastigotes in the left hind footpad. Lesion size was monitored using a vernier caliper and calculated by subtracting the size of the contralateral-uninfected footpad. Results are expressed as the mean lesion size of five mice per group (mm) \pm the standard deviation.

in footpad thickness, observed 5 and 9 weeks after infection, were statistically significant ($P < 0.05$).

Promastigote growth rates in vitro. Since changes in the kinetics of in vitro parasite growth are classically associated with changes in parasite virulence, five *L. major* isolates displaying different levels of virulence in the experimental model were selected and used for in vitro growth experiments: (i) two high virulence isolates (MHOM/SY/94/Abdou, a Syrian isolate, and MHOM/TN/95/GLC94, a Tunisian isolate); (ii) a Tunisian isolate with intermediate virulence in the experimental BALB/c infection (MHOM/TN/95/GLC34); and (iii) two

low virulence Tunisian isolates (MHOM/TN/95/GLC32 and MHOM/TN/94/GLC07). Promastigote growth rates were measured over 6 days of culture in liquid medium. Starting with an inoculum of 1×10^6 promastigotes/ml obtained after transformation of freshly harvested amastigotes, different growth curves were obtained (Fig. 2). Interestingly, the strains highly pathogenic in mice, MHOM/SY/94/Abdou and MHOM/TN/95/GLC94, although collected from distinct geographical areas, exhibited the most rapid growth, reaching a peak density of, respectively, 104×10^6 and 81×10^6 promastigotes/ml on day 4 of culture. In contrast, the less virulent isolates, MHOM/

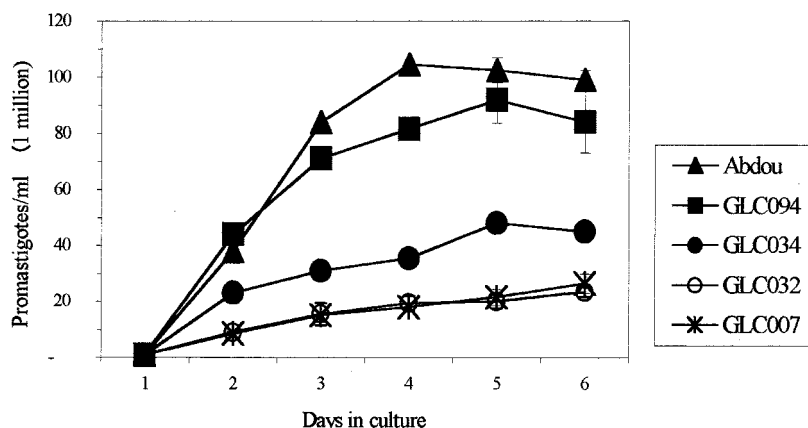


FIG. 2. Growth kinetics of five *L. major* isolates: MHOM/SY/94/Abdou and MHOM/TN/95/GLC94 (high virulence), MHOM/TN/95/GLC34 (intermediate virulence), and MHOM/TN/95/GLC32 and MHOM/TN/94/GLC07 (low virulence). Cultures were initiated in liquid medium at 10^6 promastigotes/ml. Results are reported as the mean \pm the standard deviation of two samples derived from two separate experiments.

TN/95/GLC32 and MHOM/TN/94/GLC07, grew very slowly and reached a maximum cell density of almost 26×10^6 promastigotes/ml on day 6. The MHOM/TN/95/GLC34, which had an intermediate pathogenicity, also showed a growth rate which was intermediate between those expressed by the high or the low virulence strains. The growth characteristics of these strains were reproducible over two different experiments.

Three *L. major* isolates, MHOM/SY/94/Abdou, MHOM/TN/95/GLC34, and MHOM/TN/95/GLC32, selected on the basis of their different pathogenicity in BALB/c mice (respectively, high, intermediate, or low) were used for further experiments.

In vitro bone marrow macrophage infection with *L. major* amastigotes. Infection of MBMM derived from BALB/c mice was used to test for differences in the rate of macrophage infection with the three *L. major* isolates selected. Two hours after addition of the parasites, cells were washed to remove unbound amastigotes, and the rate of macrophage infection was measured between 0.5 and 72 h of culture by examining five central fields for each enumeration. Results are expressed as the percentage of infected macrophages (Fig. 3A) and the mean number of amastigotes detectable within one infected macrophage (Fig. 3B). At every measurement time (0.5, 2, 24, and 72 h), the highly virulent *L. major* isolate MHOM/SY/94/Abdou displayed the highest infectivity for MBMM compared to the intermediate isolate ($\chi^2 = 40.53$, $P < 0.00001$; $\chi^2 = 5.18$, $P = 0.0227$; $\chi^2 = 2.2$, $P = 0.138$; $\chi^2 = 11.7$, $P = 0.000623$) or the low virulence isolate ($\chi^2 = 54.25$, $P < 0.00001$; $\chi^2 = 16.04$, $P = 0.000062$; $\chi^2 = 0.53$, $P = 0.465$; $\chi^2 = 11.7$, $P = 0.000623$). Furthermore, the parasite burden in the infected macrophages was significantly higher in macrophages infected with the MHOM/SY/94/Abdou isolate compared to those infected with the other isolates (MHOM/SY/94/Abdou versus MHOM/TN/95/GLC34, pooled t value = 13.47, $P < 0.00001$; MHOM/SY/94/Abdou versus MHOM/TN/95/GLC32, pooled t value = 13.88, $P < 0.00001$).

Cytokine response in mice infected with different *L. major* isolates. Cytokine production was comparatively analyzed for mice infected with *L. major* isolates MHOM/SY/94/Abdou, MHOM/TN/95/GLC32, or MHOM/TN/95/GLC34. Experiments were performed 5 weeks after inoculation of 2×10^6

amastigotes. Cell suspensions prepared from the lymph node draining the inoculation site were in vitro stimulated by *Leishmania* antigen, and then IL-4 and IFN- γ were measured in the supernatants by ELISA. Differences in both IFN- γ (Fig. 4A) and IL-4 (Fig. 4B) levels were found. Interestingly, the most virulent strain, MHOM/SY/94/Abdou, induced the highest IL-4 levels compared to MHOM/TN/95/GLC34 and MHOM/TN/95/GLC32 (Kruskal-Wallis test = 5.68; $P = 0.0583$), although pairwise comparisons showed that the difference did not reach significance. In contrast, MHOM/TN/95/GLC32 and MHOM/TN/95/GLC34 isolates induced the highest, though not significant, levels of IFN- γ production compared to MHOM/SY/94/Abdou isolate (Kruskal-Wallis test = 4.13; $P = 0.1265$).

Early IL-4 mRNA expression in susceptible and resistant mice following *L. major* infection. Since the highest susceptibility to *L. major* of BALB/c mice compared to C57BL/6 mice was previously reported to be associated with an early 16-h burst of IL-4 expressed by the former (24), IL-4 mRNA levels within the draining lymph nodes were compared in mice infected for 16 h with the *L. major* strains MHOM/SY/94/Abdou, MHOM/TN/95/GLC34, and MHOM/TN/95/GLC32. Groups of five mice for each strain were sacrificed in order to investigate their early IL-4 response. As controls, normal mice (BALB/c and C57BL/6) or saline-injected mice were also used. No IL-4 mRNA was detected in the lymph nodes of control mice (data not shown).

As shown in Fig. 5, the mRNA transcript levels for the HPRT gene (control) were similar in BALB/c and C57BL/6 mice. Interestingly, in BALB/c mice IL-4 mRNA levels, which reflect the early cytokine response, were higher in mice infected with the highly virulent strain MHOM/SY/94/Abdou. In contrast, the low virulence strain MHOM/TN/95/GLC32 induced lower levels of IL-4 mRNA transcripts in BALB/c mice. As expected, the early 16-h IL-4 burst was undetectable in resistant C57BL/6 mice.

LACK sequence analysis. Taking into account that the early IL-4 peak production is related to the recognition of a single *L. major* antigen LACK by a select set of $V\beta_4/V\alpha_8$ T cells (19, 23) and considering the different levels of IL-4 mRNA production in mice infected with different pathogenic strains, we

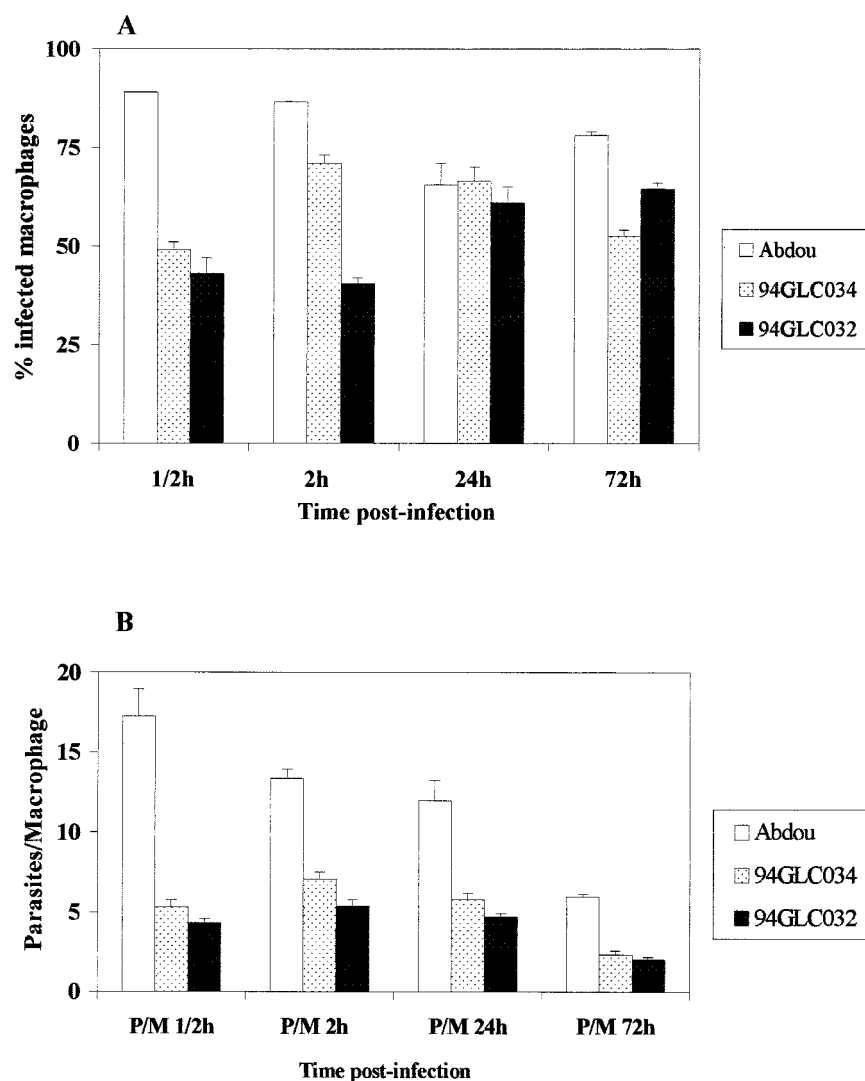


FIG. 3. In vitro phagocytosis and survival of three *L. major* isolates in macrophages. MBMM were infected with amastigotes derived from either one of three isolates at a ratio of four parasites/macrophage. At the indicated time, the fraction of infected macrophages (A) and the mean number of intracellular parasites per macrophage (B) were determined. Results are reported as the mean \pm the standard deviation of duplicate samples.

asked whether differences in LACK sequences might be responsible for differences of the early IL-4 expression.

The cDNA region coding for the peptide 155 to 196 including the 18-amino-acid I-A^d T-cell epitope was amplified by PCR and then sequenced in four strains of *L. major* with different pathogenic behaviors (two high-virulence strains, MHOM/SY/94/Abdou and MHOM/TN/95/GLC94, and two low-virulence strains, MHOM/TN/95/GLC32 and MHOM/TN/94/GLC07). Sequence comparison between these strains and the reference strain *L. major* LV39 (35) does not reveal any differences.

RT-PCR analysis of virulence gene mRNA transcripts expressed in *L. major* wild strains. It has been established that *Leishmania* parasites express, either constitutively or in a developmentally regulated manner, several molecules required for virulence. In order to determine the genetic basis for the virulence variability observed between wild strains, we analyzed them for their mRNA expression of eight virulence

genes, namely, the lipophosphoglycans *lpg-1* and *lpg-2* (4, 8, 47, 51), the LACK antigen (6, 19, 44), the kinetoplastid membrane protein KMP-11 (36, 37), the gene B protein G-BP (11, 29), the heat shock protein Hsp-100 (16, 52), and the cysteine proteinases *Cpb* (33) and *Cpc* (2).

The semiquantitative analysis was performed for three strains classified as highly virulent (MHOM/SY/94/Abdou, MHOM/TN/95/GLC67, and MHOM/TN/95/GLC94) and two strains of low virulence (MHOM/TN/94/GLC07 and MHOM/TN/95/GLC32). The comparative mRNA analysis was performed using either the amastigotes (Fig. 6A) or the stationary-phase promastigotes (Fig. 6B). The expression of α -tubulin mRNA was used as control, and samples containing similar levels of α -tubulin mRNA transcripts were run in electrophoresis. No significant difference in the abundance of the mRNA transcripts for the eight genes tested was observed between the five strains at either the amastigote or the promastigote stage.

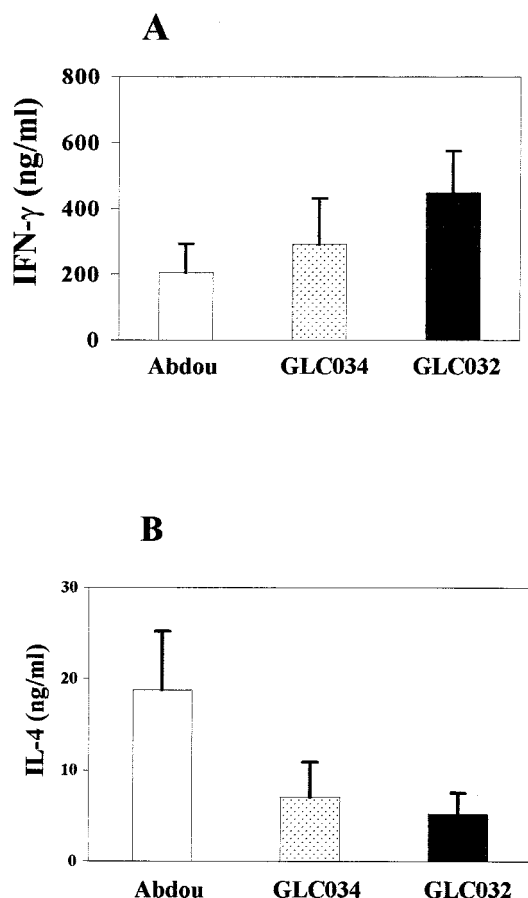


FIG. 4. Cytokine production by mononuclear cells from draining lymph nodes obtained at week 5 postinfection from mice inoculated with *L. major* isolates. Lymph node cells were incubated with *Leishmania* antigens, and the culture mediums collected after 72 or 48 h were assayed by ELISA for IFN- γ (A) or IL-4 (B), respectively. Results are reported as the mean \pm the standard deviation of four mice infected for 5 weeks with three different *L. major* isolates.

DISCUSSION

The present study has tried, using the BALB/c mouse model of *L. major* infection, to analyze the variability in the natural virulence of *L. major* strains isolated in the field from human lesions, the reservoir, or the vector. Although these isolates are likely heterogeneous (i.e., nonclonal), we decided to use unseparated parasite populations in order to stick with the actual situation prevailing in the field.

We compared the pathogenicity in BALB/c mice and studied some biologic properties of 19 *L. major* isolates collected from Tunisia ($n = 12$) or from the Middle East ($n = 7$). All strains established in Tunisia, except one, were studied shortly after isolation by using a standardized culture procedure composed of 2 days in NNN (two to four passages) and adaptation in RPMI-10% FCS for 2 to 4 days. Seventeen strains were obtained from human lesions, one strain from the reservoir animal, and one strain from the sandfly vector.

Our results show that the 19 strains expressed a significant variability in the pathogenicity induced in BALB/c mice. More importantly, these differences could be reproduced in independent experiments and could be demonstrated not only with

promastigotes (data not shown) but also with amastigotes. The latter result rules out the possibility that the detected differences might merely reflect variable levels of in vitro metacyclogenesis reached by the different strains. Some isolates were able to induce large lesions, as commonly reported in BALB/c mice. However, and quite unexpectedly, several isolates induced only very small lesions that were not different in size from those usually obtained when *L. major* parasites are injected in the genetically resistant C57BL/6 mouse strain. However, we did not observe the total regression of the lesion and the apparent cure, which characterize the experimental disease in C57BL/6 mice.

Our results allowed us to classify the 19 strains into three virulence levels: high, intermediate, or low. Interestingly, the proportion of strains that fell within each group appeared different when strains from Tunisia were compared to those from the Middle East. Thus, all strains from the Middle East were classified as expressing high ($n = 5$) or intermediate ($n = 2$) virulence whereas the majority of those from Tunisia were expressing low ($n = 4$) or intermediate ($n = 6$) virulence, with only two strains from this country expressing high virulence. Whether these differences in the experimental pathogenicity, induced by strains from North Africa or the Middle East, are germane to differences in the clinical severity of disease in humans is unknown; however, it is worth noting that ZCL due to *L. major* has globally a more benign evolution in Tunisia than in the Middle East (3, 21). Thus, at El Guettar, where almost all Tunisian strains used in this study were collected, a prospective follow-up of ZCL previously showed that 65% of cutaneous lesions had spontaneously improved after 2 weeks and almost all of them had completely healed after 15 weeks (28).

In a similar study, Almeida et al. (1) showed that different strains of *L. amazonensis* isolated from patients with distinct clinical forms (cutaneous versus visceral) promote different courses of infection in BALB/c mice. However, strains isolated from patients with visceral leishmaniasis are less pathogenic than those isolated from patients with cutaneous leishmaniasis. Considering the number of uncontrolled parameters that govern human infection with *Leishmania* parasites, it is hazardous

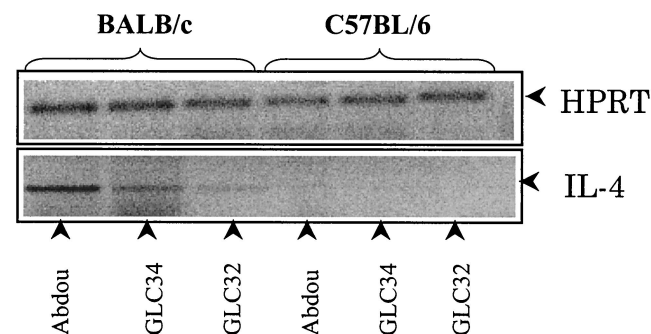


FIG. 5. RT-PCR analysis of the early burst of IL-4 mRNA in lymph nodes of C57BL/6 and BALB/c mice 16 h after footpad injection of 2×10^6 amastigotes of either MHOM/SY/94/Abdou, MHOM/TN/95/GLC34, or MHOM/TN/95/GLC32 *L. major* strains. The level of HPRT transcripts in each sample was used to control for differences in mRNA concentration and the efficiency of cDNA synthesis. Reproducible results were obtained in three separate experiments.

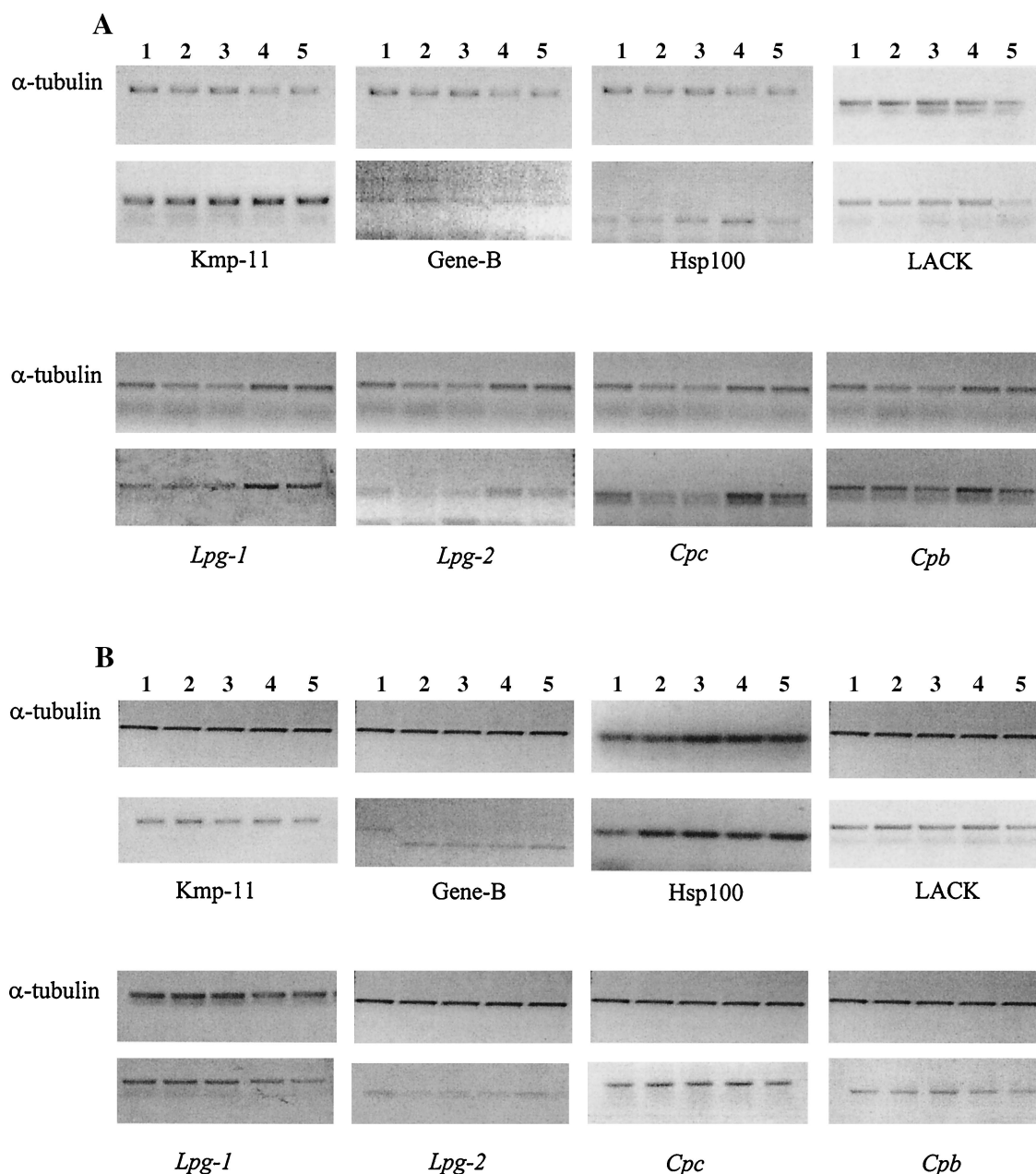


FIG. 6. RT-PCR analysis of the mRNA transcripts of eight virulence genes in *L. major* strains expressing either high virulence (MHOM/SY/94/Abdou, lanes 1; MHOM/TN/95/GLC94, lanes 2; and MHOM/TN/95/GLC67, lanes 3) or low virulence (MHOM/TN/95/GLC32, lanes 4; and MHOM/TN/94/GLC07, lanes 5). The level of α -tubulin transcripts in each sample served as control for mRNA integrity and content. The levels of specific mRNA were analyzed in parasites at the amastigote (A) or the stationary-phase promastigote (B) stage.

to try to find a link between the clinical expression of human infection and parasite pathogenicity in the experimental model.

In the present study, three strains expressing, respectively, high, intermediate, and low virulence levels were selected and studied for their capacity to infect bone marrow macrophages and to grow in vitro in liquid medium. We found that the high-virulence strain was characterized by a significantly higher ability to infect MBMM, especially during the first 2 h after infection, and to survive at higher density within the infected macrophages. Parasites from strain MHOM/SY/94/Abdou were about two to three times more abundant within macro-

phages than strains with lower virulence. Similarly, the kinetics of growth in liquid medium of the three strains was strikingly different. The higher the virulence, the faster the in vitro growth. Thus, the parasite density reached by strain MHOM/SY/94/Abdou after 2 days of culture was higher than that reached by the least virulent strain after 6 days of culture. The strain with intermediate virulence showed also an intermediate kinetics of growth. Moreover, the plateau phase reached significantly higher levels with the virulent strain compared to the two others.

Our results also show that isolates with higher virulence tend

to generate more IL-4 and less IFN- γ than low-virulence strains when lymph node cells are stimulated with LTA and tested at week five. These data suggest that the former strains tend to induce a stronger TH2 response than the latter. Similar results were previously reported with *Leishmania* clones isolated in vitro (25).

It seems unlikely that the differences in the cytokine patterns reflect variation in the proportion of lymphocytes within the draining lymph nodes, notably due to a polyclonal B-cell proliferation. Indeed, besides a slight increase in the proportions of B cells (B-220) that reach 13 to 27% of total lymphocytes, 4 to 5 weeks after experimental infection with the different *L. major* isolates, the proportion of T-cell subsets (CD3, CD4, and CD8) were not significantly different between mice infected with the different *L. major* strains (data not shown).

Interestingly, we found that the isolates with the highest virulence were also able to induce a higher early IL-4 response (measured at 16 h postinfection). Previous studies have demonstrated that this early IL-4 response is an important determinant of the TH2 dominance in BALB/c mice and can be attributed to the activation of a V α ₈/V β ₄ CD4⁺ T lymphocyte subset by an immunodominant epitope of the LACK *Leishmania*-specific antigen (23, 24). Whether the variability in the virulence phenotype reported in our study is the result of a variable capacity of the isolates to activate the LACK-specific V α ₈/V β ₄ subset is unknown. However, it is worth noting that these isolates were found to express identical sequences of the epitope of the LACK antigen encompassing amino acids 156 to 173 as assessed by sequence analysis of PCR amplicons generated from these isolates.

Several parasite molecules have been shown to play an important role in *Leishmania* virulence. Alteration in the structure of lipophosphoglycan, a major surface component of the parasite glycocalyx, was reported to correlate with alteration of the parasite virulence. Thus, ricin-resistant avirulent clones are defective in LPG synthesis and revert to virulence when incubated with LPG (20, 30, 46). Similarly, reversion to virulence after passing *Leishmania* strains into BALB/c mice is associated with higher expression of LPG (46). The higher virulence of metacyclic promastigotes compared to the procyclic parasites also correlates with changes in LPG structure (39). LPG acts as a receptor for attachment to macrophage cell membrane, as a cofactor for survival within the phagolysosome as well as a modulator of several macrophage cytokines. Considering these important functional roles of LPG in host-parasite interactions, it was considered a major determinant of parasite virulence, although this view has been recently challenged (17).

Several key enzymes in LPG synthesis have been identified (32). In our study we did not detect any significant differences at the level of mRNA expression in *lpg-1* or *lpg-2* transcripts between *L. major* strains expressing variable levels of virulence. Obviously, these data cannot rule out a role for other possible LPG genes or a nontranscriptional level of regulation. Moreover, one may observe that LPG is downregulated at the amastigote stage which was used throughout this study to detect differences in strain virulence. Therefore, other factors acting at the amastigote stage are more likely implicated. Several other proteins have been described as virulence factors: non-LPG mannose-rich glycosylated molecules (i.e., GIPLs

and PPGs) (18), gene B protein (11, 29), *Cpc* and *Cpb* cysteine proteinases (2, 33), KMP-11 (36, 37), and HSP-100 (16, 52).

In the present study, no differences in the level of mRNA transcripts of these genes could be detected between *L. major* wild strains expressing variable levels of virulence. As for *lpg-1* and *lpg-2* genes, these results cannot rule out subtle changes or nontranscriptional levels of regulation for these genes. However, they prompted us to use a broader screening technique than the gene candidate approach to screen for mRNA species differentially expressed among these isolates (26). Several clones have already been identified, and one of them, which we fully identified, corresponds to an important component of the enzymatic machinery of the parasite (Y. Ben Achour et al. unpublished data). Studies are in progress to identify the molecular basis of virulence variability among *L. major* wild strains.

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